J. Embryol. exp. Morph. Vol. 40, pp. 83–89, 1977
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Alkaline phosphatase activity in the preimplantation mouse embryo

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SUMMARY

Alkaline phosphatase (AP) activity has been assayed in frozen sections of preimplantation mouse embryos by an azo-dye cytochemical method. The results indicate that during preimplantation mouse development AP activity is first expressed between the 8- and 16-cell stages and develops in all cells by the late morula stage. During blastocyst formation AP activity is lost or greatly reduced in trophoblast cells while activity is maintained in the inner cell mass.

INTRODUCTION

Blastocyst formation in the mouse typically occurs between the 32- and 64-cell stages (McLaren & Bowman, 1973), and is marked by the appearance of a fluid-filled blastocoel cavity and two morphologically distinct cell types: the outer cells or trophoblast and the inner cell mass (ICM). These two cell populations demonstrate different developmental pathways, the trophoblast forming the ectoplacental cone and giant cells, and the ICM forming the embryo proper subsequent to implantation. Trophoblast and ICM have been shown to differ in protein composition (Van Blerkom, Barton & Johnson, 1976; Martin, Smith & Epstein, unpublished results). However, with the exception of the acid and alkaline phosphatases (Mulnard, 1955, 1965), little information is available concerning possible enzymic differences between these two cell populations at the blastocyst stage.

Since the initial report of alkaline phosphatase (AP) activity in the preimplantation mammalian embryo (Mulnard, 1955), the point in early development at which AP activity first appears and its intra-embryonic localization have been the subjects of some controversy. Some investigators, utilizing whole-mounts of rat and mouse embryos and the Gomori cytochemical technique (Gomori, 1952), reported AP activity in the central cells of 16-cell embryos and in ICM cells of the blastocyst although trophoblast cells were negative (Mulnard, 1955; Izquierdo, 1967; Ortiz, Carranza & Izquierdo, 1969; Izquierdo & Ortiz, 1975). However, other investigators utilizing both Gomori and azo-dye

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(Burstone, 1962) techniques on whole-mount embryos have described the appearance of AP activity in late 4- to 8-cell embryos, with increasing activity up to the blastocyst stage, but no differences in the intra-embryonic distribution of reaction product between inner and outer cells of any stage of rat or mouse preimplantation development (Rodé, Damjanov & Škreb, 1968; Solter, Damjanov & Škreb, 1973; Izquierdo & Marticorena, 1975).

Biochemical assay of whole mouse embryos developed *in vivo* (Izquierdo & Marticorena, 1975) has detected initial AP activity at the 8-cell stage, an increase to a maximum at 8- to 16-cell stages, and a gradual decline subsequently. Mouse blastocysts developed *in vivo* produce no evidence of AP activity on electrophoretic analysis (Sherman, 1972).

Although Mulnard (1965) has referred to the use of sectioned embryos, all previously published cytochemical investigations of the distribution of AP activity within preimplantation mouse embryos have been conducted on whole-mount embryos. It has been argued (Rodé *et al.* 1968; Solter *et al.* 1973) that the higher activity levels reported for inner cells may be due to cell overlap in the center of whole-mounts and/or other artifacts. In order to circumvent these difficulties this study utilizes frozen sections of mouse embryos to examine the development of AP activity during the preimplantation period.

MATERIALS AND METHODS

Preimplantation mouse embryos (ICR strain) were flushed from the oviducts of superovulated pregnant females at the 2-cell stage and cultured in a modified embryo culture medium (Biggers, Whitten & Whittingham, 1971). Embryos of 2-cell, 8-cell, early morula (compacted, 8–16 cells), late morula (16–32 cells) and blastocyst stages were assayed for AP activity by an azo-dye technique (Burstone, 1962). Primary oocytes obtained by puncturing ovarian follicles, unfertilized eggs from superovulated unmated females and late morulae and blastocysts developed *in vivo* were similarly assayed. An *in vitro* blastocyst outgrowth and an ICM isolated by immunosurgery from a mouse blastocyst (Solter & Knowles, 1975) were also assayed for AP activity.

Samples of 20 to 30 embryos of a given developmental stage were placed within an ant pupa case (Mintz, 1971), fixed with 2% paraformaldehyde in 0·1 M phosphate buffer (pH 7·1) for 10 min and briefly rinsed in phosphate-buffered saline (pH 7·1–7·2). In addition, a group of blastocysts was fixed for 1 h to analyze the effects of increased fixation. The pupa case was frozen by a 5–15-sec immersion in liquid nitrogen-cooled 2-methyl butane (Matheson, Coleman & Bell, Manufacturing Chemists) and subsequent immersion in liquid nitrogen for 1–3 min. Sections of approximately 8 μm were cut on an International cryostat and mounted on coverslips previously subbed with a solution of 0·5% gelatin and 0·05% chromium potassium sulfate (Pappas, 1971).

A simultaneous coupling azo-dye technique (Burstone, 1962) was utilized

for the cytochemical demonstration of AP activity because of its precise localization of final reaction product and applicability to frozen sections (Pearse, 1968). The AP reaction medium consisted of 0·08% fast Blue RR and 0·02% Na-α naphthyl acid phosphate in 0·2 M Tris-HCl buffer (pH 9·4). Control medium contained no substrate. Following Millipore filtration (0·22 μm), drops of experimental or control medium were placed over tissue sections and incubated at 37°C for 15 min. To determine the effects of extended incubation times, blastocysts and late morulae were also incubated in reaction medium for periods of 30 min, 1 h, 2 h or 4 h. Coverslips were then rinsed in phosphate-buffered saline, mounted on microscope slides with a glycerol–water (1:1) solution, observed under bright-field microscopy and photographed.

RESULTS

All preimplantation stages prior to the early morula, including primary oocytes and unfertilized eggs, were negative for AP activity. Reaction product, which appeared as a finely granular, grey-black deposit, was first identified in some cells of early morulae (Fig. 1A). At the late morula stage, reaction product was apparent in most cells and was more dense than that in early morulae (Fig. 1B, C). Central cells did not show a more positive AP reaction than peripheral cells in either of the morula stages assayed. In blastocysts, however, there developed a marked difference in AP activity between the ICM and the trophoblast. Cells of the ICM were strongly positive in their reaction while cells of the mural trophoblast (those surrounding the blastocoel) appeared negative or at least much less positive (Fig. 1D, 1E). The level of AP activity in cells of the polar trophoblast (those overlying the ICM) could not be accurately determined due to the level of resolution allowed by the techniques utilized. In some samples polar trophoblast cells appeared to be less reactive than those of the ICM, while in others they were indistinguishable from the AP-positive cells of the ICM. Distribution of reaction product in late morulae and blastocysts developing *in vivo* was similar to that observed in the *in vitro* samples. In all cases, reaction product was more dense over the cell periphery than over the cytoplasm. This is consistent with the observation that in most cell types the majority of AP activity is localized on the plasma membrane, with areas of lesser activity existing at intracellular sites such as the nucleus and the Golgi complex (Fishman, 1974). The embryonic portion of the blastocyst outgrowth and the isolated ICM also demonstrated a positive reaction for AP activity. Control sections consistently showed no evidence of reaction product (Fig. 1F).

Extended incubation times in AP reaction medium (up to 4 h) produced a slight increase in density of reaction product but no differences in its intra-embryonic distribution in late morula and blastocyst stages. Blastocysts undergoing extended fixation (1 h) also displayed an increased density of reaction product throughout the embryo but a marked difference in intensity between ICM and trophoblast was still readily apparent.

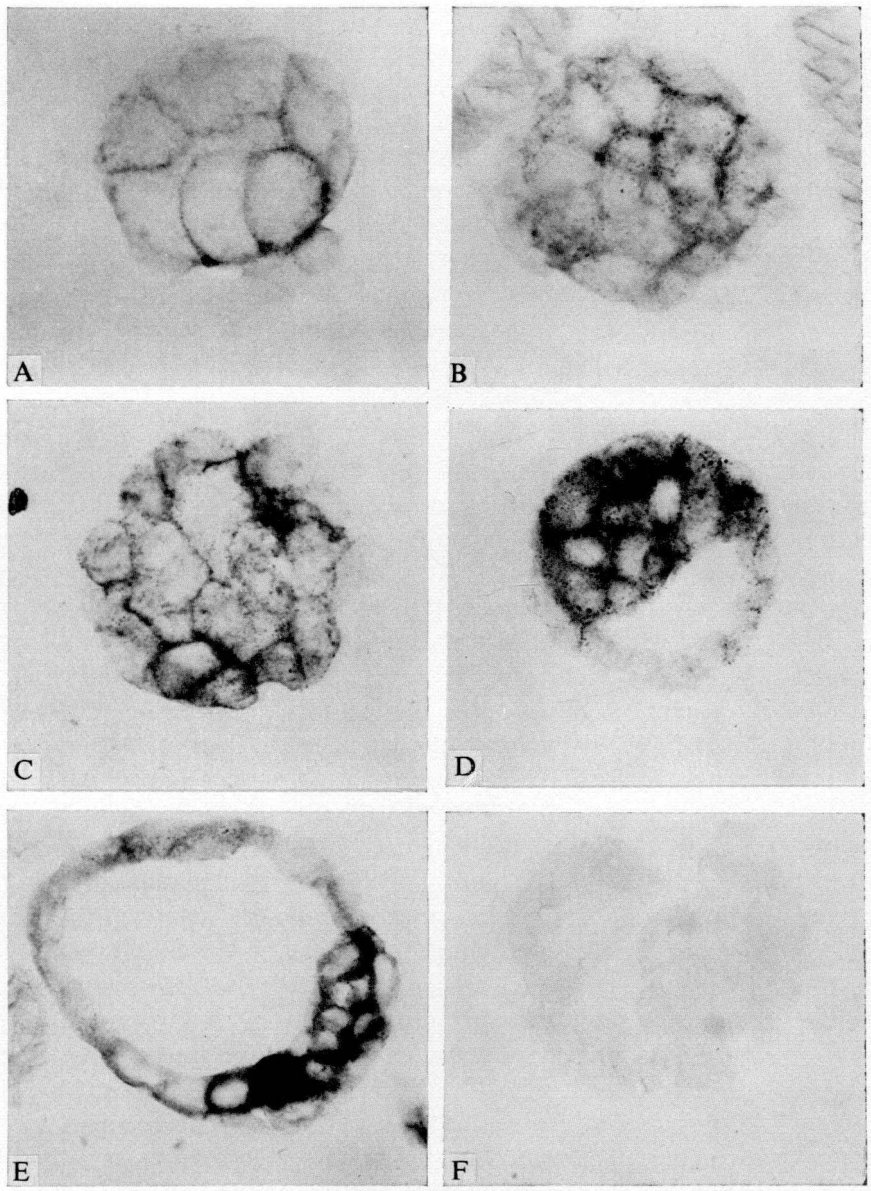

Fig. 1. Unstained 8 μm frozen sections of preimplantation mouse embryos with the cytochemical identification of alkaline phosphatase activity appearing as a dark precipitate. (A) early morula; (B) and (C) late morulae; (D) and (E) blastocysts; (F) control blastocyst. (All figures $\times 450$.)

DISCUSSION

This study demonstrates by an azo-dye technique a higher level of alkaline phosphatase (AP) activity in the ICM than in the trophoblast of sectioned preimplantation mouse embryos, whether developed *in vivo* or *in vitro*. This

observation supports previous reports (Mulnard, 1955; Izquierdo, 1967; Ortiz *et al.* 1969) of such an intra-embryonic distribution of AP activity as assayed in blastocyst whole-mounts by the Gomori cytochemical technique. Since this study utilizes sectioned embryos it is unlikely that cell overlap is responsible for the more intense staining of ICM than trophoblast as has been suggested for those studies utilizing whole-mounts (Rodé *et al.* 1968; Solter *et al.* 1973). However, the demonstration of ICM-trophoblast differences in AP activity by an azo-dye technique is in conflict with the results of previous investigations utilizing similar techniques in which no such differences were evident (Rodé *et al.* 1968; Solter *et al.* 1973; Izquierdo & Marticorena, 1975). The basis for this conflict in results is unclear.

The results of this study do not, however, reveal intra-embryonic differences in the distribution of AP activity at the morula stage as reported previously (Mulnard, 1955; Rodé *et al.* 1968; Solter *et al.* 1973). Rather, the present study supports the contentions of Solter *et al.* (1973) that AP activity is equally distributed in all cells of morulae, and that observations of more pronounced activity in inner cells of whole-mounts may in fact be due to cell overlap. In early morulae it is clear that not all blastomeres are positive for AP activity (Fig. 1A) but no regular aggregations of positively-reacting cells in the central region are apparent. Since almost all cells in late morulae are positive for AP activity (Fig. 1B, C) it is possible that negatively reacting cells of early morulae have not yet initiated synthesis or activation of AP activity.

During the process of blastocyst formation, AP activity is apparently lost or greatly reduced, specifically in the cells of the trophoblast (Fig 1D, E). Izquierdo & Marticorena (1975) have suggested that such a decrease may be indicative of trophoblast differentiation. However, the observations of relatively high levels of AP activity in trophoblast derivatives of early postimplantation embryos (Rodé *et al.* 1968; Sherman, 1972) suggest that this decrease in activity is only temporary. Alternatively, the reduction in AP activity in trophoblast might be explained by the expression of a less reactive form of the enzyme due to *de novo* synthesis or activation of a preexisting protein. Decreasing AP activity (Moog, 1965; Damjanov, Solter & Škreb, 1971; Bernstine, Hooper, Grandchamp & Ephrussi, 1973; Martin & Evans, 1975) and changing molecular forms of AP (McWhinnie & Saunders, 1966; Moog, Etzler & Grey, 1969; Pfohl, 1975) have both been reported to occur during the process of differentiation in other developmental systems.

The authors thank Dr Gail R. Martin and Dr Mitchell S. Golbus for aid in manuscript preparation and Dave Akers for photographic assistance. This study was supported by USPHS Grants GM-19527 and CA-16493 and by the Department of Anatomy, University of California, San Francisco, California.

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(Received 11 October, 1976, revised 25 January 1977)